(*NEW SERIES.*)

No. I.

SCIENTIFIC MEMOIRS

BY

OFFICERS OF THE MEDICAL AND SANITARY DEPARTMENTS

OF THE

GOVERNMENT OF INDIA.

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BY

G. LAMB, M.B., CAPTAIN, I.M.S., AND WM. HANNA, ESQ., M.B., ETC.

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IN 1884, the publication of the Scientific Memoirs by Medical Officers of the Army of India was begun. The series was intended to furnish a convenient medium for the circulation of selected original papers on scientific subjects, which it had been the custom to include in the Annual Report of the Sanitary Commissioner with the Government of India. Twelve parts of the Scientific Memoirs have been published, the last in 1901.

The collection of scientific papers in a volume, appearing periodically at considerable intervals of time, presents a serious disadvantage, since considerable delay may occur between the publication of some of the papers and the completion of the work which they describe.

To obviate this, it has been decided that in future the papers will be published as soon as possible after they are received; and the opportunity afforded by this change is taken to modify the title of the series, so that original papers on scientific subjects by all officers of the Medical and Sanitary Departments of the Government of India may be included.

B. FRANKLIN,
Surgeon-General, I.M.S.

OFFICE OF THE SANITARY COMMISSIONER
WITH THE GOVERNMENT OF INDIA;
Simla, June 30, 1902.

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Standardisation of Calmette's Anti-venomous Serum with Pure Cobra Venom:

The Deterioration of this Serum through keeping in India.

THE standardisation of an anti-venomous serum is of great practical as well as theoretical importance.

It is the practical side of the question to which we propose to confine ourselves in this paper.

The snake venom which M. Calmette [1] employs, both for the purpose of the immunisation of his horses and for the purpose of the standardisation of the serum recovered from those immune horses, is a mixture of colubrine and viperine poisons, the former making up about 80 per cent. of the mixture. A solution of this mixture is heated at about 73° C. for half an hour and then filtered. It is this mixed and heated poison which is employed at Lille for both the purposes above mentioned.

Myers [2] has correctly pointed out that as we have in all cases clinically to deal with intoxication by a pure venom, either colubrine or viperine, and also by an unheated venom, it is preferable, if the true value of this serum as a curative agent against snake-bite is to be determined, to use a pure unheated poison for its standardisation. The object then of our investigation was to ascertain, as exactly as possible, the neutralising power of Calmette's anti-venomous serum when tested with pure, fresh and carefully dried cobra poison. Further, in consequence of the observations made by us in a recent case of cobra intoxication, [3]* we have extended our investigations so as to ascertain if any deterioration of the serum takes place through keeping in this country.

In another communication we hope to be able to record the results of similar experiments made with the venom of Russell's Viper (Daboia Russellii).

The method of standardisation of the anti-venomous serum which we adopted in our experiments was the method now recognised as giving the most accurate results. It is based on the almost generally acknowledged fact, that snake poison toxin and its anti-toxin interact directly, and that this neutralisation is, so far as our knowledge goes, independent of vital activity. The evolution of this method is, briefly stated, as follows. The method which M. Calmette [4] recommends and still practises is the following:—The dose of venom (mixed and

* Reported to Government on 24th and 25th January 1901, *vide* Plague Research Laboratory's No. 130, to the address of the Secretary to the Government of India, Home Department.

heated), which will kill a rabbit of 2 kilos in weight in from 15 to 20 minutes when injected into the marginal vein of the ear, is determined; then the minimum amount of serum, which will prevent death when injected intravenously five minutes before this dose, is ascertained: this is done by experimenting with varying quantities of serum. If one cubic centimetre is able to accomplish this, M. Calmette says that the serum contains 2,000 immunity units per cubic centimetre or 20,000 per 10 cubic centimetres.

Martin of Melbourne[5] was the first to use the method of mixing the venom and serum *in vitro* before injection. In some early experiments with the poison of the Australian tiger snake (Hoplocephalus curtus) he used only a single lethal dose in estimating the curative value of Calmette's serum. However, in some later researches he recognised that this method was fallacious, inasmuch as if the amount of serum was able to neutralise even a mere fraction of the single lethal dose the animal would recover, even though the larger part of the poison remained unneutralised. He therefore recommended the employment of at least ten lethal doses.

Along with one of us, Major D. Semple, R.A.M.C.,[6] has pointed out that the following objections could be taken to Calmette's method:—

(1) that there is no direct estimation of the amount of venom which a given quantity of serum will neutralise *;

(2) that there is no estimation made of the amount of venom, the injection of which an untreated animal is capable of surviving.

Further, we showed that Calmette's test for a dose for the rabbit amounted to about three lethal doses for that animal. We thus gave a value for the serum in terms of its neutralising power and not, as in the method used at Lille, in terms of its preventive power. With such a small test dose as three lethal doses, unless careful attention be paid to the second objection mentioned above, it is evident that a much enhanced neutralising power would be given to the serum.

The next step in advance was made by Myers.[7] As previously mentioned, this observer pointed out that in order to ascertain the strength of an anti-venomous serum for practical purposes, it was *à priori* preferable to test it against a pure venom, such as pure cobra poison or pure daboia poison. He further showed that it was preferable also to use unheated venom, inasmuch as heating at the temperature employed by M. Calmette causes important changes in cobra venom, altering it considerably from the natural poison as injected by a snake. Further, Myers recommended that a cheap, small and susceptible animal be employed. He showed that mice of about 15 grammes weight answer all requirements, and recommended that these animals should be used. He further designated

* This is evident from the description of Calmette's method already stated. From this it will be seen that the standard unit which he adopts is the weight in grammes of a rabbit which 1 C. Cm. of serum can protect against a quantity of poison which would kill a control animal in 20 minutes.

Calmette's method as unscientific, inasmuch as by injecting the serum before the venom the completion of the reaction between these two bodies does not take place before they enter the blood stream. He showed that much more accurate results are attained when the poison and serum are mixed *in vitro* than by Calmette's method. Finally, he recognised the necessity of using as the test dose a multiple, and, if possible, a large multiple of the minimum lethal dose.

In the series of experiments which we have now to present, these various points were kept carefully in view.

We may preface the protocols by giving a short account of the method employed and of the general results obtained.

The animals we used were rats from 115 to 120 grammes in weight. These animals fulfil all the conditions indicated by Myers. They are cheap and the supply is abundant, while with care no difficulty is experienced in handling them. At the same time they are extremely susceptible to cobra poison, as susceptible as, if not more so than, any of the other laboratory animals. Thus for a mouse of 15 grammes Myers [8] states the minimum lethal dose of cobra venom to be 0·012 mgrm. or in other words 0·8 mgrm. per kilo. For rabbits Elliot [9] has found that a certain lethal dose of pure cobra poison injected subcutaneously is 0·7 mgrm. per kilo. In our experiments we found the minimum lethal dose of this poison for a rat of about 120 grammes was 0·04 or at the rate of 0·33 mgrm. per kilo.

Another reason for selecting rats was that horses' serum, even in large doses has no lethal action on them as it has on mice. As in the course of our experiments large doses of serum had to be given, this was an important point.

Great care was taken in choosing rats of one size for all experiments, *viz.*, of about the weight mentioned above.

The venom used was pure, fresh, unheated cobra poison. This was collected from a number of living, full-sized, healthy cobras by "milking" the glands after the method practised by M. Calmette. The poison was then thoroughly dried over lime and kept, till used, in an air-tight tube away from light. Before weighing it was pounded in a mortar and then again dried over sulphuric acid. A solution of this powdered venom in normal salt solution was made. The strength of this solution was 0·1 per cent. When necessary a tenfold dilution of this material was employed. All the solutions were freshly made as required, so that no deterioration of the poison was possible through keeping in solution. All injections were made in the inner side of the thigh.

Series No. I was undertaken to determine the minimum lethal dose for a rat of 115 to 120 grammes in weight.

A certain lethal dose was found to be 0·04 mgrm., and the maximum non-lethal dose was found to be 0·035 mgrm.

The remaining series of experiments were undertaken with a view of

determining the neutralising power of various samples of anti-venomous serum. These samples were of different ages and had been in India for varying periods of time. They had been stored in different places and had therefore been exposed to different degrees of heat.

The test dose of venom which was used in all cases consisted of ten times the minimum lethal dose, *i.e.*, 0·4 mgrm. This quantity was mixed *in vitro* with varying amounts of the serum to be tested. The mixture was allowed to stand at laboratory temperature (about 25° C.) for at least half an hour. All injections were made, as in the experiments detailed in series No. I, subcutaneously in the inner side of the thigh.

In calculating the amount of venom which the serum had been able to neutralise, or had failed to neutralise, the maximum non-lethal dose—*i.e.*, 0·035 mgrm.—was deducted from the test dose in all cases.

In series No. II a serum which had recently been received from Lille was used. We take this opportunity of thanking M. Calmette for his courtesy in placing this serum at our disposal.

This sample was dated 17th September 1900. It arrived in Bombay on the 14th October of the same year. Our experiments with it were made between the 10th November and the 5th December. We are justified, therefore, in considering this as perfectly fresh serum, as fresh as it would be possible to get in this country.

A reference to the protocols will show that 0·5 C. Cm. was able to prevent death, while 0·45 C. Cm. did not accomplish this. In other words 0·5 C. Cm. of this serum was able to neutralise 0·4 — 0·035 mgrm.=0·365 mgrm.; namely, 1 C. Cm. could neutralise 0·73 mgrm.

Working with a mixed and heated venom, Semple and one of us [10] found that 1 C. Cm. of fresh serum was able to neutralise 1·5 mgrm. of this mixed poison. This is the same value which M. Calmette attributes to his serum, when this is expressed in terms of the neutralising power of the serum. Comparing these three results, we see at once, therefore, the importance of testing the serum against a pure unheated venom and not with a mixed and heated poison.*

The serum employed to perform the experiments detailed in series No. III was obtained by purchase from Messrs. Phillips & Co., Chemists, Bombay. It bore the date 8th May 1900. The vendors informed us that they had received it from Europe about the end of August 1900, and that it had not been out of Bombay. Our experiments were made between the 5th and 12th December 1900.

* This comparison, as well as other similar comparisons to be detailed later on, is made on the assumption that all specimens of anti-venomous sera sent out from Lille have, when fresh, the same neutralising power. There can be no doubt, however, that such a desirable result cannot practically be obtained. It is probable, therefore, that a certain proportion of the differences in the neutralising power of the sera with which we experimented may be due to initial differences in this neutralising power.

It will be seen therefore that this serum had been in Bombay for about three months. One of these months, October, is one of the hottest months of the year.

A reference to the protocols will show that 0·6 C. Cm. was able to prevent death, while 0·5 C. Cm. could not accomplish this. It is evident therefore that 1 C. Cm. of this serum was able to neutralise 0·61 mgrm.

This result would appear to point to a slight deterioration of the serum having taken place through keeping in Bombay, even for the short time mentioned above.

The serum used in series No. IV of our experiments was obtained from the Natural History Society of Bombay. We take this opportunity of thanking Mr. Phipson, the Honorary Secretary of this Society, for his kindness in placing it at our disposal.

This sample of serum was not dated. Mr. Phipson, however, thought that it had been in his possession for about four years. On reference to Lille, M. Calmette informed us that he did not know the exact age of the serum, but that it was at least more than three years old. From the protocols it will be seen that although one rat, which received 0·75 C. Cm. of serum, recovered after being very ill, the quantity of serum necessary to prevent a fatal result with certainty was 1·25 C. Cm. It is evident therefore that 1 C. Cm. of this serum was able to neutralise only 0·29 mgrm. of pure cobra venom.

The fifth series of experiments detailed in the protocols was made with serum which was sent to us by Captain Mitchell of Nasik. In view of the results obtained, the history of this sample of serum is interesting. It was originally the property of the Bombay Natural History Society, and was got from Europe at the same time as the serum used in series No. IV of our experiments; it was therefore more than three years old. Soon after its arrival in Bombay it was sent to Nasik. It was kept there till Captain Mitchell sent it to us in October, 1900. Our experiments with this serum were made between the 16th October and 1st November 1900.

A reference to the protocols will show that 0·8 C. Cm. was able to prevent death when our test dose was employed. In other words 1 C. Cm. of this serum was able to neutralise with certainty 0·46 mgrm. of pure cobra poison.

For series No. VI of our experiments the sample of serum was obtained from Lieutenant-Colonel G. H. D. Gimlette, I.M.S., then Residency Surgeon, Indore.

This sample was dated 17th October 1896. Its history was as follows:—It was imported to India by Messrs. Smith, Stanistreet & Co., Chemists, Calcutta, arriving in this country about the end of August 1899. It was sent to Indore on the 6th September of the same year. Colonel Gimlette despatched it to Bombay at the end of November 1900, and our experiments were completed

between the 29th November and 3rd December. It will be seen, therefore, that for over a year it lay at Indore and was thus subjected to the great heat of that place during one hot season.

A reference to the protocols will show that 1·7 C. Cm. of this serum failed to neutralise the test dose, while 2 C. Cm. were able to accomplish this. The rat which received this latter quantity was ill for a short time. We may take it, therefore, that 2 C. Cm. was the amount which could neutralise just over nine lethal doses (0·365 mgrm.), or in other words that 1 C. Cm. was able to neutralise only 0·18 mgrm. of venom.

The experiments detailed in series No. VII of the protocols were made with a serum received from Mr. Hankin of Agra. This sample bore the date 17th September 1895. It had been stored in Agra for over four years. It was received in Bombay on the 17th October 1900, and our experiments with it were completed between the 27th November and 10th December.

A glance at the protocols will show that 2·5 C. Cm. of this serum, *i.e.*, the largest amount used, were not able to prevent death when our test dose of ten lethal doses was employed. From this it is evident that 1 C. Cm. of this Agra serum could not neutralise even 0·14 mgrm. of poison. The serum can, therefore, be said to have lost all neutralising power for practical purposes.

The serum employed in the eighth and last series of experiments detailed in the protocols was received from Lieutenant W. G. Liston, I.M S., Civil Surgeon, Ellichpur, Berar. It was dated 7th November 1896.

Lieutenant Liston had purchased it from Lille in September 1898, and had brought it with him to India the following month. During the hot seasons of 1899 and 1900 the serum had been subjected to the heat of Berar at two stations, *viz.*, Hingoli and Ellichpur. It was sent to Bombay in December 1900. The experiments made by us were completed between the 10th and 14th December. From the protocols it will be seen that 2 C. Cm. of this serum failed to neutralise the test dose. We can therefore conclude that 1 C. Cm. was not able to neutralise 0·18 mgrm. of venom. It is unfortunate that no more serum was available to complete this series. It is, however, evident that, like the Agra serum, it had lost for all therapeutical purposes its power to neutralise pure cobra poison.

We may now pass on to consider some of the practical bearings which the data collated above and detailed in the protocols have on the standardisation of anti-venomous serum and on the therapeutics of cobra-poisoning.

Firstly, our experiments conclusively prove Myers's statement that it is necessary in standardising this serum to use a pure unheated poison. Both in Calmette's method of standardisation and in the method followed by Semple and one of us, a mixed venom containing about 80 per cent. of colubrine poison was employed. The solution of this mixture was heated at 73° C. for half an hour and then

filtered. The conclusion arrived at in both cases was the same, *viz.*, that 1 C. Cm. of serum was able to neutralise 1·5 mgrm. of this venom. As this mixture contained only a fifth part of viperine poison it appeared justifiable to conclude that 1 C. Cm. of serum would be able to neutralise at least about 1·2 mgrm. of pure cobra poison. Our experiments, however, have shown that such a hypothesis is not borne out by fact, and that 1 C. Cm. of serum can only neutralise about 0·7 mgrm. of pure cobra venom.*

Secondly, it is evident that any attempt to calculate the therapeutic dose of the serum can only be made when the exact measure of its power of neutralising a natural poison has been ascertained. Let us consider this point in some detail.

Calmette† recommends that from 10 to 20 C. Cm. be given as the dose in all cases of bites from poisonous snakes. We shall see later that such a dose would probably be insufficient in some cases of cobra bite.

Semple and one of us [11] calculated that 15 C. Cm. were sufficient to save a patient, if this quantity were injected into the blood before the lapse of such period of time as might be required for the absorption of the lethal dose of venom. This calculation, however, was made on the following assumptions :—

(1) That the minimum lethal dose of cobra venom for a man of 60 kilos weight was, as estimated by Fraser,[12] about 31 mgrms., and that consequently we may take 30 mgrms. as the maximum non-lethal dose for an average man.

(2) That a full-sized cobra can inject at each bite a quantity of venom which in the dried state amounts to from 30 to 45 mgrms. These amounts have been demonstrated experimentally by Calmette.[13]

To save a man bitten by a cobra, injecting the maximum quantity possible, we should therefore have to give an amount of serum capable of neutralising 45—30 mgrms.=15 mgrms. of cobra poison.

(3) That 1 C. Cm. of serum was capable of neutralising 1 mgrm. of pure cobra venom. This figure was arrived at by assuming that pure unheated cobra poison was not more than a third more deadly than the mixed venom we employed. This mixture, it will be remembered, contained 80 per cent. of colubrine poison, and 1 C. Cm. of serum was able to neutralise 1·5 mgrm. of it.

It is evident, therefore, that were these three assumptions correct, 15 C. Cm. of serum would be a safe dose to employ in all cases of cobra bite.

Our experiments, however, have demonstrated that 1 C. Cm. of serum is only capable of neutralising about 0·7 mgrms. of pure cobra venom, while Myers [14] from his experiments on mice makes the quantity neutralised by 1 C. Cm. even less than this, *viz.*, 0·6 mgrms.

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* The serum which we used in these experiments, although newly arrived in India, had necessarily been subjected for a short time to the heat of the voyage out—*viz.*, in the Red Sea and the Indian Ocean. A certain deterioration may thus have taken place. This, however, is likely to account for only a limited proportion of this great difference.

† This is the dose prescribed in the printed instructions sent out with every bottle of serum.

Now, basing the calculation on this fact, *viz.*, that 1 C. Cm. of serum can neutralise from 0·6 to 0·7 mgrms. of cobra venom, and on the first two assumptions stated above, we arrive at the conclusion that instead of 15 C. Cm. of serum, a dose of from 22 to 25 C. Cm. would have to be given to prevent death from the bite of a full-sized cobra.

As we have already stated, one of the assumptions on which the above calculation is founded is, that the minimum lethal dose of cobra venom for a man is as much as 31 milligrammes. This assumption is based partly on the fact, experimentally shown by Calmette [15] and other observers, that for such animals as the guinea-pig, the rabbit, the dog, the monkey and the ass, the larger the animal the greater is the amount of cobra venom, weight for weight, required to cause death. This rule, however, does not hold good for all species of animals. Thus Myers [16] has shown that the minimum lethal dose of cobra poison for a mouse of 15 grammes in weight is 0·012 mgrms. If the rat were as susceptible to this poison as is the mouse, the lethal dose for a rat of 120 grammes in weight would be 0·096 mgrms. We have, however, seen above that the minimal lethal dose for a rat of this size is only 0·04 mgrms., namely, that the rat is at least twice as susceptible to cobra venom as is the mouse.

In view of this fact it would seem expedient, in estimating the dose of anti-venomous serum to be given in a case of cobra bite, to base our calculations on the assumption that man is at least as susceptible as the most susceptible animal with which experiments have been made, *viz.*, the rat. Assuming then that man is as susceptible as the rat, it is evident that the minimum lethal dose for a man of 60 kilos weight would be about 20 mgrms., and the maximum dose which he may naturally be able to resist, say 19 mgrms. We can therefore calculate that if a cobra had injected the maximum amount of venom possible, *viz.*, 45 mgrms. (an assumption which it would be necessary to make in every case of snake bite), the quantity of serum required to merely save the life of any one receiving this injection would be the amount which was capable of neutralising at least 45 — 19 mgrms. = 26 mgrms. This amount of fresh serum,—namely, serum, 1 C. Cm. of which was able to neutralise about 0·7 mgrms. of cobra venom,—would be about 37 C. Cm. It is interesting in this connection to point out that Calmette [17] himself has arrived at the conclusion that the minimum lethal dose of cobra venom for a man is only about 10 mgrms. Working on this latter hypothesis, it is evident that it would require about 50 C. Cm. of serum to save a patient who had received the maximum quantity of venom which a cobra can inject.

Our contention that 15 C. Cm. of serum is quite an inadequate dose for the treatment of a case of cobra bite, receives corroboration from a consideration of the accidental case which occurred in this laboratory a short time ago.[18] *

The only treatment employed in this case was the subcutaneous injection of

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* *Vide* foot-note on page 1.

anti-venomous serum. Immediately after the accident 18 C. Cm. of old serum were injected. This was the serum obtained from the Bombay Natural History Society, the neutralising power of which was tested in the experiments detailed in series No. IV of the protocols. From a reference to the protocols it will be seen that 1 C. Cm. of this serum was able to neutralise 0·29 mgrm. of pure cobra venom.

It follows, then, that 18 C. Cm. of such serum was equivalent to 7·5 C. Cm. of fresh serum, that is, of serum 1 C. Cm. of which was capable of neutralising 0·7 mgrm. of venom. Our patient, therefore, received as a preliminary dose a quantity of serum equivalent to 7·5 C. Cm. of a prefectly fresh serum. In spite of this injection, well-marked general symptoms referable to the central nervous system set in about 2½ to 3 hours after the accident. The case in all probability would have had a fatal ending had not a second injection of fresh serum been given before the symptoms had progressed too far.

Now in this case the poison from one gland only was injected. We can therefore conclude that 15 C. Cm. of fresh serum would have been quite inadequate to have prevented the onset of symptoms of intoxication and probably would not have prevented death had the patient received the quantity of poison contained in both glands.

Shortly after the onset of symptoms 10 C. Cm. of comparatively fresh serum were injected. This was serum got from Messrs. Phillips & Co., *vide* series No. III in protocols. We have seen that 1 C. Cm. of this serum was capable of neutralising 0·61 mgrm. of cobra poison. The dose which was injected was therefore, equivalent to 8·7 C. Cm. of perfectly fresh serum, such as was used in the experiments detailed in series No. II of the protocols. Our patient, therefore, received in all a quantity of serum equivalent to 16 C. Cm. of perfectly fresh material.

All symptoms in this case, in which the bite was inflicted by only one fang, soon passed off after the second injection of serum. Since, however, the second dose was given after some of the poison had already joined on to the central nervous system, it is for *à priori* reasons necessary to conclude that a smaller dose would have sufficed, if the whole quantity had been injected before the onset of any symptoms. On the other hand, it must be remembered that the cobra which inflicted this bite had been in captivity for some months and would not consequently yield as much poison as an ordinary snake in nature would do. For it is an observation of common occurrence in this laboratory that a cobra newly caught will yield from 20 to 30 large drops of poison, while after he has been a captive for some time this quantity will have diminished to from 6 to 10 drops and in time to *nil*.

From what we have stated above it is evident that the question of the dose of serum to be injected in cases of cobra bite turns upon factors which cannot be

directly experimentally determined—*viz.* :—(1) upon the amount of poison which the snake has injected; and (2), upon the amount of cobra venom which an average man can survive.

In view of the fact, however, that in the treatment of any case of cobra bite we must always estimate the dose of antivenene to meet the possibility of the snake having injected the maximum quantity possible, *i.e.*, about 45 mgrms., and in view of the fact that man may be as susceptible, weight for weight, as the rat or perhaps more susceptible, it seems to us from the considerations put forward above that a dose of from 30 to 35 C. Cm. of fresh serum, or the equivalent of this, should be given in every case of cobra bite. A larger dose would, of course, be necessary if symptoms had set in before the treatment was begun. At any rate the history of this case shows that a dose of about 15 C. Cm. would be quite inadequate in most cases of cobra bite.

We may now pass on, in conclusion, to consider briefly the question of the deterioration which anti-venomous serum undergoes by keeping in India. The data bearing on this subject are contained in detail in the protocols, series No. II to No. VIII inclusive. These data may be briefly summarised in tabular form as follows :—

Series in Protocols.	Age of serum.	Length of time in India.	Where kept in India.	Neutralising power.	Remarks.
No. II	2 months	1 month	Bombay	1 C. Cm. neutralised 0.73 mgrm.	This serum may be regarded as perfectly fresh serum.
No. III	7 months	3 months	Bombay	1 C. Cm. neutralised 0.61 mgrm.	
No. IV	Over 3 years	About 3 years	Bombay	1 C. Cm. neutralised 0.29 mgrm.	
No. V	Over 3 years	About 3 years	Nasik and Bombay.	1 C. Cm. neutralised 0·46 mgrm.	It is uncertain how long this sample had been in Bombay and how long in Nasik.
No. VI	4 years	1¼ years	Indore	1 C. Cm. neutraiised 0·18 mgrm.	
No. VII	5 years	Over 4 years	Agra	1 C. Cm. failed to neutralise 0·14 mgrm.	
No. VIII	4 years	2¼ years	Bombay, and Berar.	1 C. Cm. failed to neutralise 0·18 mgrm.	Bombay 0 months, Berar 1¾ years.

The protective power ascribed by M. Calmette to all samples of sera sent out from Lille is 20,000 units per 10 C. Cm. We may, therefore, assume that all brews when fresh possess practically the same neutralising power (*vide* foot note, page 4). A study of the above table shows that a progressive deterioration takes

place from keeping the serum in India. Thus it will be noticed, on the one hand, that while the serum which was seven months old and had been in India for only three months (No. III) already showed a slight diminution in its neutralising power; on the other hand, the sera which had been kept for some years in the Berars (Hingoli and Ellichpur), *i.e.*, No. VIII, and in Agra, *i.e.*, No. VII, had practically lost all neutralising power, and the sera which had been stored at Indore, Bombay, and Nasik had deteriorated to a considerable extent, but had not completely lost all neutralising power.

Let us consider for a moment the climatic conditions as regards temperature which prevail at these different places.

In the cold season Agra and the Berars have a comparatively low temperature; but for three or four months of the year the heat in these districts is excessive, the maximum temperature in the shade being seldom below 105° F. and often reaching 110° F. and over.

Indore also has a severe hot weather and a comparatively cool cold season, a similar climate in fact to that of Berar, although, perhaps, the maximum temperature does not reach so high as in the other two cases.

The greater neutralising power of the serum which had been kept at Indore compared with the Berar and Agra samples, can be accounted for by the fact that the former sample had been subjected to this great heat for only one hot season, while the Berar and Agra sera had been stored in these districts during two and four hot weathers, respectively.

The results obtained with sera Nos. IV and V are especially interesting. Both these sera were of the same age and were imported to Bombay at the same time. One sample, *viz.*, No. V, had been stored for a considerable time at Nasik after Bombay. Now the temperature of Nasik during the cool season of the year is much lower than that of Bombay. On the other hand, during the hot months, March to May inclusive, the maximum shade temperature of Nasik is only slightly lower than that of Bombay, while the minimum temperature is much lower. On the whole, therefore, we may take it that the serum which had been kept at Nasik had not been subjected to nearly such a high mean temperature during the year as had that serum which had been stored in Bombay. In consequence, the neutralising power of the former was more than a half greater than the neutralising power of the latter.*

We may conclude then from these data that anti-venomous serum undergoes a progressive and fairly rapid deterioration when stored in hot climates, and that this deterioration is greater and more rapid the higher the mean temperature to which it is subjected.

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* Experiments are at present in progress in this Laboratory to ascertain, if possible, the causes of this difference,—such as the effect of heating on the *poison* as regards the amount of serum required to neutralise a given quantity before and after heating, etc.

The practical bearings of the results of these observations are so patent as to require no further discussion.

Protocols.

Series No. I.—Experiments to determine the minimum lethal dose of pure cobra venom for a rat of about 118 grammes.

The poison used was carefully dried pure fresh cobra venom. It was unheated. A 0·1 per cent. solution in sterile normal saline solution was made. Ten-fold and hundred-fold dilutions of this original solution were used when necessary. The weights refer to the original dried venom.

All rats were carefully selected about 118 grammes weight. The injections were made subcutaneously in the inner side of the thigh.

Animal.	Amount of dried venom in Milligrammes.	Result
Rat 1	0·1	Died within 2 hours.
Rat 2	0·09	Died within 2 hours.
Rat 3	0·08	Died within 2 hours.
Rat 4	0·07	Died within 3 hours.
Rat 5	0·06	Died within 3 hours.
Rat 6	0·05	Died within 3 hours.
Rat 7	0·05	Died within 3 hours.
Rat 8	0·045	Very ill for 24 hours; died in 4 days.
Rat 9	0·04	Died in 3¼ hours.
Rat 10	0·04	Died in 2¼ hours.
Rat 11	0·035	Ill for 18 hours; recovered.
Rat 12	0·035	Ill for 6 hours; recovered.
Rat 13	0·03	Slightly ill for 3 hours; recovered.
Rat 14	0·03	No symptoms.
Rat 15	0·025	No symptoms.
Rat 16	0·02	Slight symptoms; recovered.
Rat 17	0·01	No symptoms.
Rat 18	0·005	No symptoms.

From the above table it appears, then, that 0·04 mgrm. is a certain lethal dose, and that 0·035 mgrm. is a dose which a rat of this size can survive, *i.e.*, a maximum non-lethal dose.

In the series of experiments No. II to No. VIII, detailed hereafter, ten lethal doses,—namely, 0·4 mgrm.—were mixed *in vitro* with varying amounts of sera of different ages and which had been kept under different conditions of temperature. The mixtures were allowed to stand at laboratory temperature (about 25° C.) for at least half an hour, and were then injected subcutaneously in the inner

side of the thigh. Carefully selected rats of about 118 grammes weight were used in all cases. In calculating the amount of venom which the serum had been able to neutralise or had failed to neutralise, the maximum non-lethal dose —namely, 0·035 mgrm.—was deducted in all cases.

These series are as follow:—

Series No. II.—Experiments to ascertain the amount of venom which 1 C. Cm. of fresh serum newly imported was capable of neutralising.

The serum used in this series of experiments was received from Lille in October, 1900. It was dated 17th September 1900. The experiments were completed between the 10th November and 5th December.

Animal.	Amount of dried venom in Milligrammes.	Amount of serum in C. Cm.	Result.
Rat 1 . . .	0·4	0·15	Died in 5 hours.
Rat 2 . . .	0·4	0·2	Died in 10¾ hours.
Rat 3 . . .	0·4	0·2	Died in 7½ hours.
Rat 4 . . .	0·4	0·2	Died in 2¾ hours.
Rat 5 . . .	0 4	0·2	Died within 48 hours.
Rat 6 . . .	0·4	0·25	Died in 2¾ hours.
Rat 7 . . .	0·4	0·25	Died within 17 hours.
Rat 8 . . .	0·4	0·25	Died within 5 hours.
Rat 9 . . .	0·4	0·3	Very ill for 48 hours; recovered.
Rat 10 . .	0·4	0·3	Died in 5 hours.
Rat 11 . . .	0·4	0·3	Died in 2¾ hours.
Rat 12 . . .	0·4	0·3	Died within 5 days.
Rat 13 . . .	0·4	0·35	Died within 48 hours.
Rat 14 . . .	0·4	0·35	Died within 48 hours.
Rat 15 . . .	0·4	0·35	Died in 17 hours.
Rat 16 . . .	0·4	0·4	Died within 40 hours.
Rat 17 . . .	0·4	0·4	Ill for 5 days; recovered.
Rat 18 . . .	0·4	0·4	Died within 48 hours.
Rat 19 . . .	0·4	0·45	Died within 3 days.
Rat 20 . . .	0·4	0·45	Died after 8 days.
Rat 21 . .	0·4	0·5	No symptoms.
Rat 22 . . .	0·4	0·5	Ill for 2 days; recovered.
Rat 23 . . .	0·4	0·6	Ill for 2 days; recovered.
Rat 24 . . .	0·4	0·7	No symptoms.

From the above series we can conclude that 0·45 C. Cm. of this serum failed to neutralise with certainty 0·4 — 0·035 mgrm.=0·365 mgrm.; while 0·5 C. Cm. could neutralise this amount. In other words, 1 C. Cm. was able to neutralise 0·73 mgrm.

SERIES NO. III.—Experiments to ascertain the amount of venom which 1 C. Cm. of comparatively fresh serum was capable of neutralising.

The serum used in this series was dated 8th May 1900. It was purchased from Messrs. Phillips & Co., Chemists, Bombay, who had received it from England about the end of August. The experiments were completed between the 5th and 12th December. The serum had, therefore, been in Bombay during one of the hottest months of the year, *viz.*, October.

Animal.	Amount of dried venom in Milligrammes.	Amount of serum in C. Cm.	Result.
Rat 1 . . .	0·4	0·2	Died in 2 hours.
Rat 2 . . .	0·4	0·3	Died in 2 hours.
Rat 3 . .	0·4	0·4	Died within 42 hours.
Rat 4 . . .	0·4	0·5	Died within 42 hours.
Rat 5 . . .	0·4	0·5	Died within 46 hours.
Rat 6 . .	0·4	0·6	Ill for one day; recovered.
Rat 7 . . .	0·4	0·6	No symptoms.
Rat 8 . . .	0·4	0·7	No symptoms.
Rat 9 . .	0·4	0·7	No symptoms.
Rat 10 . . .	0·4	0·8	No symptoms.

From the above series we can conclude that 0·5 C. Cm. of this serum failed to neutralise 0·4 — 0·035 mgrm.=0·365 mgrm., while 0·6 C. Cm. could neutralise this amount at least,—namely, that 1 C. Cm. was able to neutralise 0·61 mgrm.

SERIES NO. IV.—Experiments to ascertain the amount of venom which 1 C. Cm. of a serum, which had been in Bombay for over three years, was capable of neutralising.

This serum had been in the possession of the Secretary of the Bombay Natural History Society for at least three years. Calmette informed us that it was at least three years old, probably more.

Animal.	Amount of dried venom in Milligrammes.	Amount of serum in C. Cm.	Result.
Rat 1 . . .	0·4	0·25	Died in 1½ hours.
Rat 2 . . .	0·4	0·5	Died in 1½ hours.
Rat 3 . . .	0·4	0·75	Died in 3 hours.
Rat 4 . . .	0·4	0·75	Very ill for 48 hours; recovered.
Rat 5 . . .	0·4	0·85	Died within 36 hours.
Rat 6 . . .	0·4	1·0	Died in 6½ hours.
Rat 7 . . .	0·4	1·0	Died in 9 hours.
Rat 8 . . .	0·4	1·0	Ill; recovered.
Rat 9 . . .	0·4	1·15	Died after 3 days.
Rat 10 . . .	0·4	1·25	No symptoms.
Rat 11 . . .	0·4	1·5	No symptoms.

From the above series of experiments we can conclude that 1·15 C. Cm. of this serum failed to completely neutralise 0·4 — 0·035 mgrm.=0·365 mgrm., while 1·25 C. Cm. did neutralise this amount at least. Therefore, 1 C. Cm. of this serum was able to neutralise 0·29 mgrm.

Series No. V.—Experiments to ascertain the amount of venom which 1 C. Cm. of a serum of the same age as that used in Series No. IV, but which had been kept during most of the time in a comparatively cool place, was capable of neutralising.

The serum used in this series of experiments was got from Captain Mitchell of Nasik. It was imported to India at the same time as the serum used in the experiments detailed in series No. IV.

After being stored in Bombay it was sent to Nasik. The exact date when this happened cannot be ascertained, as no record was kept of the event. It was received at the laboratory from Nasik in October 1900. The experiments were completed between 16th October and 13th November.

Animal.	Amount of dried venom in Milligrammes.	Amount of serum in C. Cm.	Result.
Rat 1 . . .	0·4	0·25	Died within 3½ hours.
Rat 2 . . .	0·4	0·25	Died within 3 hours.
Rat 3 . . .	0·4	0·25	Died within 7 hours.
Rat 4 . . .	0·4	0·3	Died within 4 hours.
Rat 5 . . .	0·4	0·35	Died within 4½ hours.
Rat 6 . . .	0·4	0·4	Died within 4 hours.
Rat 7 . . .	0·4	0·45	Died within 5 hours.
Rat 8 . . .	0·4	0·5	Died within 14 hours.
Rat 9 . . .	0·4	0·5	Died within 4½ hours.
Rat 10 . . .	0·4	0·5	Very ill for 24 hours; recovered.
Rat 11 . . .	0·4	0·55	Died within 40 hours.
Rat 12 . . .	0·4	0·6	Died within 50 hours.
Rat 13 . . .	0·4	0·65	Died within 54 hours.
Rat 14 . . .	0·4	0·7	Died within 50 hours.
Rat 15 . . .	0·4	0·7	Ill; recovered.
Rat 16 . . .	0·4	0·75	No symptoms.
Rat 17 . . .	0·4	0·75	Died within 8 days.
Rat 18 . . .	0·4	0·8	No symptoms.
Rat 19 . . .	0·4	1·0	No symptoms.
Rat 20 . . .	0·4	1·25	No symptoms.
Rat 21 . . .	0·4	1·5	No symptoms.

From the above series of experiments we can conclude that 0·75 C. Cm. of this serum did not completely neutralise 0·4 — 0·035 mgrm.=0·365 mgrm., but that 0·8 C. Cm. was able to do this. It is evident, therefore, that 1 C. Cm. of this sample could neutralise 0·46 mgrm.

SERIES NO. VI.—Experiments to ascertain the amount of venom which 1 C. Cm. of serum received from Lieutenant-Colonel Gimlette, Indore, was able to neutralise.

This serum was dated 17th October, 1896. At the time of our experiments it was, therefore, five years old. It was imported to India in September 1899. It arrived in Indore the same month, and was kept there till November 1900, when it was sent to Bombay.

The experiments were completed between 27th November and 3rd December.

Animal.	Amount of dried venom in Milligrammes.	Amount of serum in C. Cm.	Result.
Rat 1 . . .	0·4	0·5	Died in 2 hours.
Rat 2 . . .	0·4	0·6	Died in 2 hours.
Rat 3 . . .	0·4	0·7	Died in 1¼ hours.
Rat 4 . . .	0·4	0·8	Died in 1½ hours.
Rat 5 . . .	0·4	0·9	Died in 2 hours.
Rat 6 . . .	0·4	1·0	Died in 1¾ hours.
Rat 7 . . .	0·4	1·1	Died in 20 hours.
Rat 8 . . .	0·4	1·2	Died in 20 hours.
Rat 9 . . .	0·4	1·3	Died in 2¾ hours.
Rat 10 . . .	0·4	1·4	Died in 7½ hours.
Rat 11 . . .	0·4	1·5	Died in 19 hours.
Rat 12 . . .	0·4	1·6	Died in 90 hours.
Rat 13 . . .	0·4	1·7	Died within 67 hours.
Rat 14 . . .	0·4	2·0	Ill for 2 days; recovered.

From the above series of experiments we can conclude that 2 C. Cm. was the minimum amount of serum which was able to neutralise 0·4 — 0·035 mgrm.= 0·365 mgrm. In other words, 1 C. Cm. was able to neutralise 0·18 mgrm.

Series No. VII.—Experiments to ascertain the amount of venom which 1 C. Cm. of serum received from Mr. Hankin, Agra, was able to neutralise.

This sample of serum bore the date 17th September 1895.

Mr. Hankin informed us that he had kept no record of the exact date when he received this sample of serum, but that he had little or no doubt that he received it in the year in which it was prepared. We may, therefore, take it that this serum had been in Agra for over four years.

Animal.	Amount of dried venom in Milligrammes.	Amount of serum in C. Cm.	Result.
Rat 1 . . .	0·4	0·7	Died in 5 hours.
Rat 2 . . .	0·4	0·8	Died in 5 hours.
Rat 3 . . .	0·4	0·9	Died in 8 hours.
Rat 4 . . .	0·4	1·0	Died within 48 hours.
Rat 5 . . .	0·4	1·1	Died in $3\frac{3}{4}$ hours.
Rat 6 . . .	0·4	1·2	Died in 9 hours.
Rat 7 . . .	0·4	1·25	Died in 19 hours.
Rat 8 . . .	0·4	1·3	Died in 9 hours.
Rat 9 . . .	0·4	1·4	Died in 9 hours.
Rat 10 . . .	0·4	1·4	Died in 30 hours.
Rat 11 . . .	0·4	1·5	No symptoms.
Rat 12 . . .	0·4	1·5	Died within 18 hours.
Rat 13 . . .	0·4	1·5	Died in 38 hours.
Rat 14 . . .	0·4	1·6	Died within $22\frac{1}{2}$ hours.
Rat 15 . . .	0·4	1·6	Died in 18 hours.
Rat 16 . . .	0·4	1·7	Died within 18 hours.
Rat 17 . . .	0·4	1·7	Died in $16\frac{3}{4}$ hours.
Rat 18 . . .	0·4	1·8	Died within 18 hours.
Rat 19 . . .	0·4	1·9	Died within 18 hours.
Rat 20 . . .	0·4	2·0	Died in 24 hours.
Rat 21 . . .	0·4	2·0	Died within 17 hours.
Rat 22 . . .	0·4	2·25	Died within 17 hours.
Rat 23 . . .	0·4	2·5	Died in 46 hours.

From the above series of experiments we can conclude that 2·5 C. Cm. of this serum could not neutralise 0·4 — 0·035 mgrm.=0·365 mgrm. It is evident, therefore, that 1 C. Cm. could not neutralise even 0·14 mgrm.

Series No. VIII.—Experiments to ascertain the amount of venom which 1 C. Cm. of serum received from Lieutenant W. G. Liston, I.M.S., Ellichpur, Berar, was capable of neutralising.

This serum was dated 7th November 1896. It was purchased from Lille by Lieutenant Liston in September 1898. It was imported to India the following month. For six months it was kept in Bombay, but during the hot seasons of 1899 and 1900 it was exposed to the great heat of Berar. It was sent to Bombay in December, 1900. The experiments were completed between the 10th and 14th of December, 1900.

Animal.	Amount of dried venom in Milligrammes.	Amount of serum in C. Cm.	Result.
Rat 1 . . .	0·4	0·5	Died within 3½ hours.
Rat 2 . . .	0·4	0·8	Died within 3½ hours.
Rat 3 . . .	0·4	1·0	Died within 3½ hours.
Rat 4 . . .	0·4	1·25	Died in 22 hours.
Rat 5 . . .	0·4	1·5	Died within 42 hours.
Rat 6 . . .	0·4	1·5	Died within 50 hours.
Rat 7 . . .	0·4	1·75	Died within 48 hours.
Rat 8 . . .	0·4	2·0	Died within 42 hours.

From the above series of experiments we can say that 2 C. Cm. of this serum failed to neutralise 0·4 — 0·035 mgrm.=0·365 mgrm.—namely, that 1 C. Cm. could not neutralise 0·18 mgrm.

Notes and References.

(1) Lancet, May 19th, 1900, p. 1433.
(2) Lancet, May 19th, 1900, p. 1433.
(3) Lancet, January 5th, 1901, p. 25.
(4) Annales de l'Inst. Pasteur, 1897, p. 225.
(5) Intercolonial Medical Journal of Australasia, August 20th, 1897. '*Ibid*' April 20th, 1898.
British Medical Journal, October 15th, 1898, p. 1120.
(6) British Medical Journal, April 1st, 1899, p. 781.
(7) *Loc. Cit.*
(8) *Loc. Cit.*
(9) British Medical Journal, February 10th, 1900, p. 309.

(10) *Loc. Cit.* In this investigation the serum was tested both by mixing the serum and poison *in vitro* before injection, and by injecting the serum before the venom. Practically identical results were got in each case.

(11) *Loc. Cit.*

(12) British Medical Journal, April 18th, 1896, p. 957.

(13) Calmette, 'Le Venin des Serpents,' 1896.

(14) *Loc. Cit.*

(15) *Loc. Cit.*

(16) *Loc. Cit.*

(17) *Loc. Cit.*

(18) Lancet, January 5th, 1901, p. 25.